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Soluble NTPDase: An additional system of nucleotide hydrolysis in rat blood serum

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Abstract

The participation of a nucleoside triphosphate diphosphohydrolase in the nucleotide hydrolysis by rat blood serum was evaluated. Nucleoside triphosphate diphosphohydrolase and phosphodiesterase are enzymes possibly involved in ATP and ADP hydrolysis. The specific activity of the phosphodiesterase activity (using thymidine 5′-monophosphate *p*-nitrophenyl ester as substrate) was 4.92 ± 0.73 (mean ± SD, n = 10) nmol *p*-nitrophenol. min^{-1}. mg^{-1} protein and the specific activities for ATP and ADP were 1.31 ± 0.37 (mean ± SD, n = 7) and 1.36 ± 0.25 (mean ± SD, n = 5) nmol Pi. min^{-1}. mg^{-1} protein, respectively. A competition plot demonstrated that ATP and ADP hydrolysis occurs at the same active site. The effect of suramin and phenylalanine on ATP, ADP and thymidine 5′-monophosphate *p*-nitrophenyl ester hydrolysis was investigated. The results were opposite considering the hydrolysis of ATP and ADP and that of the substrate marker for the enzyme phosphodiesterase. These results are indicative of the presence of, at least, two enzymes participating in the serum nucleotide hydrolysis. The presence of cAMP did not affect the hydrolysis velocity of ATP and ADP, while thymidine 5′-monophosphate *p*-nitrophenyl ester hydrolysis was inhibited by cAMP by approximately 47%, suggesting that the hydrolysis of the ATP and ADP, under our assay conditions, occurs at a different site from the phosphodiesterase site. Both enzyme activities, in the rat blood serum, may be involved in the modulation of the nucleotide/

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nucleoside ratio in the circulation, serving an in vivo homeostatic and antithrombotic function. In addition, the phosphodiesterase may act on DNA or RNA liberated upon tissue injury and/or cell death.

Keywords: NTPDase; Apyrase; ATP-ADP hydrolysis; ATPDase; PDEase; Rat blood serum

Introduction

The roles of the adenine nucleotides (ATP, ADP and AMP) and their nucleoside derivative adenosine (Ado) as compounds with opposite effects are well established. Accordingly, ATP is vasoconstrictor and may be cytotoxic, while ADP causes platelet aggregation. In contrast, Ado produced by nucleotide degradation is vasodilator, inhibits platelet aggregation and presents neuromodulator effects (Soslau and Youngprapakorn, 1997; Frassetto et al., 1993). It is known that protection of the heart from ischaemia or hypoxic episodes could be achieved with endogenous and exogenous Ado. In fact, cardiac ischaemia initiates the breakdown of ATP and ADP to Ado, which may exert beneficial effects such as mediation of vasodilatation, reduction of heart rate and contractility, inhibition of platelet aggregation, as well as protection against repetitive episodes of ischaemia (De Vente et al., 1984). Therefore, the extracellular hydrolysis of nucleotides producing Ado could be considered a very important event (Birk et al., 2002).

The level of exogenous ATP may be increased in various inflammatory and shock conditions, mainly as a consequence of nucleotide release from platelets, endothelial and blood vessel cells (Hantgan, 1984; Bodin and Burnstock, 1996; Dubyak, 2000). This rise in exogenous ATP concentration is usually accompanied by concurrent secretion of various enzymes into the intercellular space (Yegutkin and Burnstock, 2000). Thus, the measurement of the rate of ATP enzymatic hydrolysis in blood may serve as an auxiliary tool in the diagnosis of cellular damage in various pathophysiological conditions (Yegutkin, 1997).

The 5′-nucleotide phosphodiesterase (PDEase, NPPase, PC-1, EC 3.1.4.1) is a microsomal enzyme that releases mononucleoside-5′-monophosphate from the 3′-OH terminal of the nucleotides. This enzyme is known to hydrolyse not only DNA and RNA, but also UDP-galactose, NAD, cAMP, ATP and ADP. *p*-Nitrophenyl-5′-thymidine-monophosphate (*p*-Nph-5′-TMP) has been used as an artificial substrate marker for PDEase, generating *p*-nitrophenol. Of note, serum PDEase was characterized in foetal serum and is known to increase in hepatoma, being used as a marker of this liver disease (Sakura et al., 1998).

Nucleoside triphosphate diphosphohydrolase (NTPDase) is the general designation for enzymes that hydrolyse ATP, ADP and other triphospho- and diphosphonucleosides to their equivalent monophosphonucleosides and inorganic phosphate (Meyerhof, 1945), but not the other substrates of the PDEase enzyme. The presence of this enzyme activity has been well demonstrated in: a) plant tissue; b) insects; c) parasites; d) peripheral nervous system from synaptosomes of the electric ray and e) in a number of mammalian sources (Handa and Guidotti, 1996; Kettlun et al., 1992; Ribeiro et al., 1990; Sarkis et al., 1986; Battastini et al., 1998, 1991; Pilla et al., 1996; Oliveira et al., 1997).

In this study, we characterized the enzymes that may be involved in the hydrolysis of triphospho- and diphosphonucleosides in rat blood serum, and we postulated the co-existence of PDEase and NTPDase

together with a 5′-nucleotidase as constituents of the enzymatic chain able to promote ATP-ADP hydrolysis to Ado in the circulation.

Methods

Chemicals

Nucleotides, Trizma Base, EGTA sodium salt, ouabain, oligomycin, sodium azide, orthovanadate, NEM (N-ethylmaleimide), lanthanum chloride and levamisole were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All others reagents were of analytical grade.

Isolation of blood serum fraction

We used male Wistar rats of approximately 60 days old, weighting around 250 grams. Blood samples were drawn after decapitation, as described by Yegutkin, 1997, and were soon centrifuged in plastic tubes at 5000 *g* for 5 minutes at 20 °C. The serum samples obtained were then stored on ice and immediately used in the experiments.

Measurement of p-Nph-5′-TMP hydrolysis

p-Nph-5′-TMP hydrolysis was determined essentially as described by Sakura et al. (1998). The reaction mixture containing *p*-Nph-5′-TMP, as a substrate (at the concentrations indicated) in 100 mM Tris-HCl, pH 8.9, was incubated with approximately 1.0 mg of serum protein at 37 °C for 8 minutes in a final volume of 0.2 mL. The reaction was stopped by the addition of 0.2 mL of NaOH 0.2 N. Incubation times and protein concentrations were chosen to ensure the linearity of the enzymatic reaction (results not shown). The amount of *p*-nitrophenol was measured at 400 nm using an extinction coefficient of 18.8×10^{-3}/M/cm. In order to correct non-enzymatic hydrolysis, we performed controls by adding the serum after the reaction was stopped with NaOH. All samples were assayed in duplicate. Enzyme activities were expressed as nanomoles (nmol) of *p*-nitrophenol released per minute per milligram of protein.

Measurement of ATP and ADP hydrolysis

ATP and ADP hydrolysis were determined using a modification of the method described by Yegutkin (1997). The reaction mixture containing ADP or ATP as substrate (at the concentrations indicated), 112.5 mM Tris-HCl, pH 8.0, was incubated with approximately 1.0 mg of serum protein at 37 °C for 40 minutes in a final volume of 0.2 mL. The reaction was stopped by the addition of 0.2 mL of 10% TCA. The samples were chilled on ice and the amount of inorganic phosphate (Pi) liberated was measured by the method of Chan et al. (1986). Incubation times and protein concentration were chosen to ensure the linearity of the reaction (results not shown). In order to correct non-enzymatic hydrolysis, we performed controls by adding the serum after the reaction was stopped with TCA. All samples were centrifuged at 5000 *g* for 5 minutes to eliminate precipitated protein and the supernatant was used for the colorimetric assay. All samples were assayed in duplicate. Enzyme activities were expressed as nanomoles of Pi

released per minute per milligram of protein. The inhibitors used were prepared in aqueous solutions. Oligomycin, when tested, was added from a concentrated ethanol solution. The addition of ethanol alone, but at the same concentration, did not arrest the enzyme activity.

Protein determination

Protein was measured by the Coomassie Blue method according to Bradford (1976), using bovine serum albumin as standard.

Results

Characteristics of p-5′-Nph-TMP hydrolysis

The specific activity of the PDEase enzyme in the presence of 0.5 mM of substrate was 4.92 ± 0.73 (mean ± SD, n = 17) nmol *p*-nitrophenol. min^{-1}. mg^{-1} protein. In order to evaluate kinetic parameters, PDEase activity was also determined at *p*-5′-Nph-TMP concentrations ranging from 100 to 250 μM. The results indicated that the enzyme activity increased with increasing concentrations of substrate. The Eadie-Hofstee plot of the results obtained in the range of 100 to 250 μM of substrate showed that the Michaelis constant ($K_{m,\ app}$) was 229.12 ± 44.84 μM and the maximal velocity ($V_{max,\ app}$) was 8.93 ± 0.71 of *p*-nitrophenol. min^{-1}. mg^{-1} protein (mean ± SD, n = 3).

Kinetic characteristics of ATP and ADP hydrolysis

Rat serum promoted ATP and ADP hydrolysis with a specific activity of 1.31 ± 0.37 (mean ± SD, n = 7) and 1.36 ± 0.25 (mean ± SD, n = 5) nmol Pi. min^{-1}. mg^{-1} protein, respectively, in the presence of 3.0 mM of substrate. The ATPase-ADPase activities were similar with or without the addition of cations (Ca^{2+} or Mg^{2+}). However, in the presence of 15.0 mM EGTA (probably able to remove endogenous cations) the hydrolysis of both substrates could be stimulated by the addition of cations (Ca^{2+} or Mg^{2+}), although the level of hydrolysis did not reach the levels attained with the controls (without EGTA) (Fig. 1).

ATP and ADP hydrolysis were determined at ATP and ADP concentrations ranging between 0.2–5.0 mM for each substrate. The results (not shown) indicated that the enzyme activity increased with increasing concentration of both nucleotides. The Eadie-Hofstee plot of the results obtained in the range of 0.1–5.0 mM of substrates showed that the Michaelis constant ($K_{m,\ app}$) was in the range of 89–100 and 250–285 μM with a calculated maximal velocity ($V_{max,\ app}$) in the range of 1.31–1.50 and 1.79–1.90 nmol Pi. min^{-1}. mg^{-1} protein for ATPase and ADPase activities, respectively (n = 3 for each substrate).

To evaluate a possible relationship between the enzymes described in this work with classical ATPases (enzymes that preferentially hydrolyse ATP), we tested the effects of various classical inhibitors of these enzymes on ATP and ADP hydrolysis (Table 1). The following ATPase and alkaline phosphatase inhibitors did not affect the ATP and ADP hydrolysis by rat blood serum: (a) the Na^+, K^+-ATPase inhibitor: ouabain, (b) Ca^{2+}, Mg^{2+} ATPase inhibitors: NEM and lanthanum, (c) the mitochondrial ATPase inhibitors: oligomycin and sodium azide, this last at 1.0 mM and (d) a specific

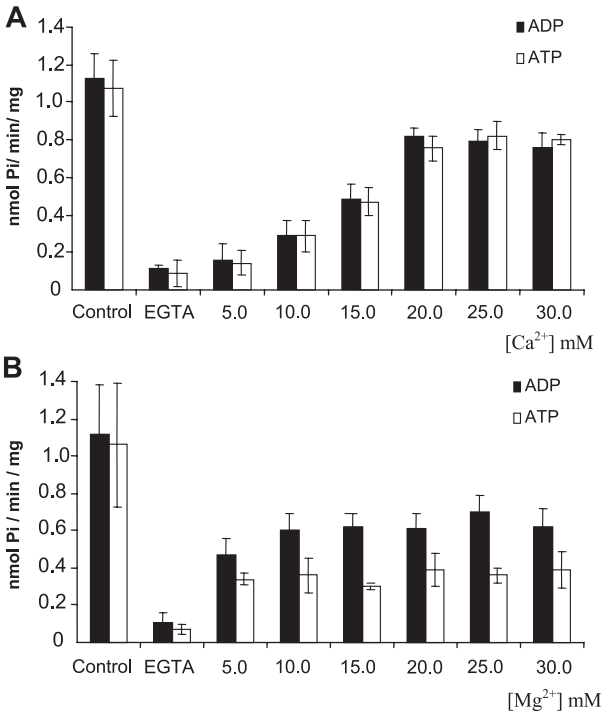

Fig. 1. Calcium (A) and Magnesium (B) ion effect on ATPase- ADPase activities. Black bars, ATPase; white bars, ADPase. The enzyme assay was carried out as described in Material and Methods. Controls (the activities were 1.31 ± 0.37 and 1.36 ± 0.25 nmol Pi. min^{-1}. mg^{-1} protein, for ATP and ADP respectively) were performed without cation addition and in the presence of nucleotides (ATP or ADP) at 3.0 mM. Other conditions were: EGTA (Control + 15.0 mM EGTA) or EGTA 15.0 mM plus a cation concentration as indicated in the figure. The same conditions were used for calcium and magnesium. The data represent three experiments ± SD with different serum preparations.

inhibitor of alkaline phosphatase: levamisole. In contrast, sodium azide, an inhibitor of various ATP diphosphohydrolases (Battastini et al., 1991; Oliveira et al., 1997; Knowles et al., 1983; Picher et al., 1994), promoted significant inhibition when tested at a concentration of 10 mM or greater. Orthovanadate (0.1 mM) is known to inhibit transport ATPases and our results demonstrated that it causes a parallel inhibition (approximately 50%) of ATPase and ADPase activities in rat blood serum. It is important to note that orthovanadate has also been described as an inhibitor of NTPDases of salivary gland, mammary gland and rat uterus (Valenzuela et al., 1989) and of rat and human placenta (Traverso-Cori et al., 1970). When we tested orthovanadate at low concentration (0.1 mM), ATPase and ADPase activities of human platelets were also inhibited in a parallel manner (Pilla et al., 1996).

To investigate if ATP and ADP hydrolysis is due to only one active site able to hydrolyse both substrates or by independent catalytic sites, the competition plot described by Chevillard et al. (1993) was performed. To assay the different combination of substrates in the competition plot, we chose a concentration at which the rate of hydrolysis was the same when either ATP or ADP were used as substrates (0.77 nmol Pi/min/mg). A sequence of mixtures with different concentrations of ATP (0.0 to 0.5 mM) and ADP (0.5 to 0.0 mM) on $P = 0$ and $P = 1$, respectively, was prepared. The curve obtained in the competition plot was a horizontal straight line, meaning that the two activities (ATP

Table 1
Effects of inhibitors on serum soluble NTPDase

Inhibitors	Concentration (mM)	% Control enzyme activity		
		ATPase	ADPase	PDEase
Ouabain	1.0	101 ± 1.1 (3)	97 ± 3.0 (3)	N.D.
NEM	1.0	97 ± 5.6 (3)	100 ± 19.5 (3)	N.D.
Lanthanum	0.1	102 ± 6.5 (3)	96 ± 10.0 (3)	N.D.
Oligomycin	2.0 μg/ml	92 ± 1.7 (3)*	91 ± 6.0 (3)	N.D.
Levamisole	1.0	107 ± 9.7 (3)	100 ± 8.5 (3)	N.D.
Sodium azide	1.0	94 ± 15.3 (3)	84 ± 12.5 (3)	N.D.
	10.0	84 ± 2.5 (3)*	79 ± 2.6 (3)*	N.D.
	20.0	76 ± 3.5 (3)*	81 ± 4.2 (3)*	N.D.
Orthovanadate	0.1	55 ± 12.6 (3)*	51 ± 6.1 (3)*	N.D.
ϕ-alanine	5.0	46.9 ± 0.06 (3)*	37.04 ± 0.1 (4)*	95 ± 0.01 (3)
Suramin	0.25	81.3 ± 0.05 (3)	107.7 ± 0.03 (4)	43 ± 0.03 (3)*
cAMP	5.0	98.78 ± 9.25 (3)	101.86 ± 12.9 (3)	46.89 ± 15 (3)*

Effects of inhibitors on ATPase, ADPase and *p*-Nph-5′-TMP activities in rat blood serum. Oligomycin was added from a concentrated ethanol solution (1% final ethanol concentration). Ethanol (1%) itself did not inhibit the enzyme activity. Results are expressed as percentages of the control activity. Data represent mean ± SD with the numbers of experiments given in parentheses. Data were analysed statistically by paired sample *t*-test and * indicates significant difference from control activity (100%) ($p < 0.05$). (N.D. = not determined).

and ADP hydrolysis) occur at the same active site on a single enzyme. Based on the preliminary experiments, the time of the enzyme assay was reduced to 30 min in order to maintain optimum kinetic conditions (Fig. 2).

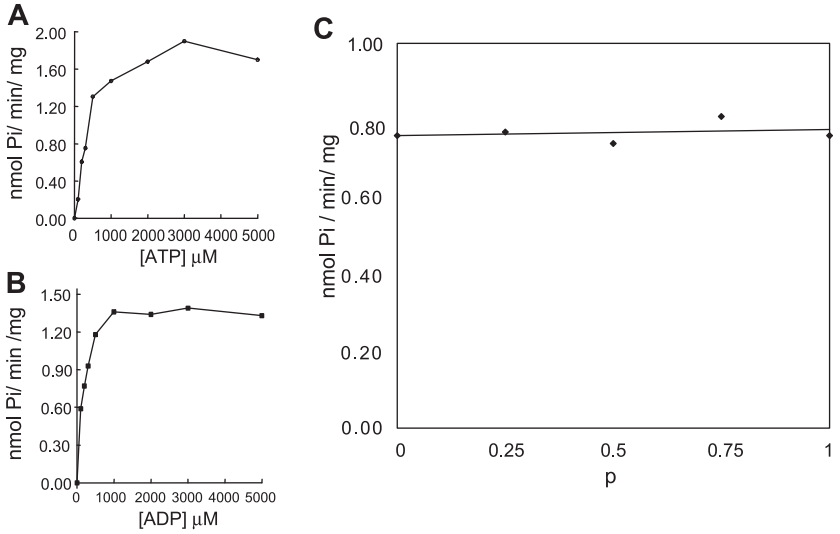

Fig. 2. The competition plot assay. (A) Substrate curve of ATP hydrolysis (100–5000 μM). (B) Substrate curve of ADP hydrolysis (100–5000 μM). (C) The competition plot: The concentration at which the velocities are the same for ATP and ADP was chosen for the Chevillard plot. The assay conditions are described in Material and Methods. The incubation time was 30 min; substrate A (ATP) at P = 0 was 0.5 mM and substrate B (ADP) at P = 1 was 0.5 mM. The data are representative of three independent experiments with different serum preparations.

As our interest in this study was to evaluate the possible co-expression of an NTPDase and of a PDEase, we searched for experimental conditions able to distinguish one from another. We observed that suramin and phenylalanine presented different effects on ATP/ADP hydrolysis and on *p*-Nph-5′-TMP. Accordingly, while phenylalanine inhibited ATP/ADP hydrolysis but not *p*-Nph-5′-TMP, suramin inhibited *p*-Nph-5′-TMP but not ATP/ADP hydrolysis (Table 1). These results are important to our hypothesis of a co-existence of a PDEase and of an NTPDase in rat serum. In a second set of experiments, the substrates ATP (3.0 mM) and ADP (3.0 mM) were incubated separately in the presence of 5.0 mM cAMP to saturate the PDEase enzyme. The results (Table 1) showed that the ATP and ADP hydrolysis is the same in the presence or absence of cAMP, suggesting that the nucleotide hydrolysis under our assay conditions occurs at a different active site from the PDEase. As expected, the incubation of 0.5 mM *p*-Nph-5′-TMP (a PDEase substrate marker), with cAMP (5.0 mM), presented an inhibition of the PDEase activity (approximately 47%) (Table 1). Thus, the hypothesis of a co-existence of a PDEase and of an NTPDase in serum of adult rats was further corroborated.

Moreover, to determine whether the enzyme activities in serum were soluble or membrane bound, we measured substrate hydrolysis both in the supernatants and pellets of a centrifugation at 4,500 × *g* for 5 minutes and of an ultracentrifugation at 100,000 × *g* for 1 hour (data not shown). Enzymatic activities in the pellets were minimal, suggesting that we are essentially dealing with soluble enzymes in the supernatants (approximately 95%).

Discussion

This investigation demonstrated that the serum of rats hydrolyses adenine nucleotides ATP, ADP and *p*-Nph-5′-TMP, suggesting the presence of the NTPDase and PDEase enzymes. The observed *p*-Nph-5′-TMP hydrolysis indicates the existence of PDEase activity in rat blood serum resulting in *p*-nitrophenol formation in vitro. The apparent K_m value of *p*-Nph-5′-TMP obtained for systemic serum agrees with the values described in the literature (Sakura et al., 1998) for the PDEase of human umbilical cord serum.

In previous studies, we investigated NTPDases in peripheral (Sarkis and Salto, 1991) and central nervous systems (Battastini et al., 1995) and platelets (Pilla et al., 1996), while in the present study we have attempted to describe this enzyme in rat blood serum. The enzyme described here has the following general properties which characterize NTPDases: a) activation by divalent metal cations such as Ca^{+2} and Mg^{+2}, in a similar manner to ATP and ADP hydrolysis, b) insensitivity to classical ATPase inhibitors (ouabain, NEM, lanthanum, oligomycin and 1.0 mM sodium azide), c) inhibition by sodium azide at high concentrations (10–20 mM), d) inhibition by orthovanadate. Furthermore, the presence of a non-specific phosphatases, which hydrolyses ATP and ADP, was excluded due to the finding that the hydrolysis of both nucleotides was not affected in the presence of levamisole (Table 1).

However, since the enzyme PDEase is available in the serum and is also able to hydrolyze ATP and ADP, we searched for evidence demonstrating the presence of an NTPDase. We observed that two inhibitors presented different effects with respect to ATP-ADP hydrolysis and *p*-Nph-5′-TMP hydrolysis. While phenylalanine did not modify the hydrolysis of *p*-Nph-5′-TMP, this aminoacid inhibited, in a parallel manner, ATP-ADP hydrolysis. Conversely, suramin affected the hydrolysis of *p*-Nph-5′-TMP,

but did not inhibit ATP-ADP hydrolysis (Table 1). This result is significant, since it indicates the presence of two enzymes with different behaviours in response to different inhibitors, suggesting the co-existence of an enzyme able to hydrolyse ATP and ADP (possibly a NTPDase) and of a PDEase.

Two other protocols were developed to evaluate our hypothesis. Firstly, we used the Chevillard plot (Fig. 2), which is a decisive method to determine if two substrates are hydrolysed at the same active site of an enzyme (Chevillard et al., 1993). The constant velocity presented by the Chevillard plot indicates that ATP and ADP are hydrolysed at only one active site. Of note, this same protocol was used to demonstrate the presence of an NTPDase enzyme in placenta (Hantgan, 1984) and in human platelets (Pilla et al., 1996). Secondly, to confirm our hypothesis, we performed experiments using cAMP, which is a classical substrate of PDEase enzyme. We observed that the ATP-ADP hydrolysis velocity is unaltered in the presence of cAMP and that *p*-Nph-5′-TMP hydrolysis was inhibited by approximately 47% in the presence of cAMP. Thus, these results provide strong evidence that the nucleotide hydrolysis occurs at a different site from the PDEase site. In agreement with this hypothesis, we demonstrated that a single convulsive injection of pentylenetetrazol increases, in rat blood serum, the ATPase-ADPase activity but not the PDEase activity (Bruno et al., 2002). Thus, in pathological conditions, different behaviours of the two serum enzymes may be observed.

In conclusion, blood serum enzymes may contribute to remove ATP and ADP and, together with a 5′-nucleotidase, may increase the concentration of Ado, a potent vasodilator. Zimmermann (1999), pointed out that PDEases might act as ‘‘guard dogs’’ to prevent subversion of the cell by destroying incoming DNA or RNA liberated upon tissue injury and cell death. Circulating nucleotides are known to be important signalling molecules, ATP acts as a vasoconstrictor and ADP is able to promote platelet aggregation. Therefore, circulating soluble ecto-enzymes, such as an NTPDase, may reduce the excess of the levels of these molecules and have an important role in maintaining normal physiology. At the same time, blood serum enzymes may work together with enzymes from platelets (Frassetto et al., 1993; Pilla et al., 1996) and from vascular wall (Yegutkin and Burnstock, 2000) to avoid spontaneous platelet aggregation and thrombus formation. The paper by Enjyoji et al. (1999) investigates the involvement of NTPDase (cd39) in these processes, a member of a family of cell surface nucleotide hydrolysing enzymes that is located at endothelial cells. Since our results were obtained in rat blood serum, it is important to consider that, upon electrical stimulation, the sympathetic nerve releases not only ATP, but also soluble enzymes (NTPDases) that may act in conjunction with membrane ectonucleotidases in the breakdown of ATP to Ado (Todorov et al., 1997). Research on this NTPDase enzyme in soluble form is of fundamental importance, since this enzyme may be used to inhibit platelet aggregation and was shown to prolong the survival of rats undergoing heterotopic cardiac xenografting from guinea pigs (Imai et al., 2000). In addition, this enzyme could be relevant as a marker for several central and peripheral diseases. NTPDase and PDEase, with different kinetic parameters, may be present in the organism as a double system for the maintenance of homeostasis. Although NTPDase and PDEase share common features, distinct characteristics exist between them. These differences suggest that the two enzymes are differently regulated and that they may function in different signalling contexts.

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